Factor Xa induces cytokine production and expression of adhesion molecules by human umbilical vein endothelial cells.

Factor Xa Induces Cytokine Production and Expression of Adhesion Molecules by Human Umbilical Vein Endothelial Cells

Nicole H. M. Senden,[1]* Trudi M. A. A. Jeunhomme,* Johan W. M. Heemskerk,†‡ Rob Wagenvoord,† Cornelis van't Veer,[2]* H. Coenraad Hemker,† and Wim A. Buurman*

Proinflammatory effects induced by the serine protease factor Xa were investigated in HUVEC. Exposure of cells to factor Xa (5–80 nM) concentration dependently stimulated the production of IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1) and the expression of E-selectin, ICAM-1, and VCAM-1, which was accompanied by polymorphonuclear leukocyte adhesion. The effects of factor Xa were blocked by antithrombin III, but not by the thrombin-specific inhibitor hirudin, suggesting that factor Xa elicits these responses directly and not via thrombin. IL-1α and TNF-α were not implicated, since neither the IL-1 receptor antagonist nor a TNF-neutralizing Ab could suppress the factor Xa responses. Active site-inhibited factor Xa and factor Xa depleted from γ-carboxyglutamic acid residues were completely inactive. The effector cell protease receptor-1 (EPR-1) seems not to be involved since anti-EPR-1 Abs failed to inhibit cytokine production. Moreover, neither the factor X peptide Leu^{83}-Leu^{88}, representing the inter-epidermal growth factor sequence in factor Xa that mediates ligand binding to EPR-1, nor the peptide AG1, corresponding to the EPR-1 sequence Ser^{123}-Pro^{137} implicated in factor Xa binding, inhibited the factor Xa-induced cytokine production. In conclusion, these findings indicate that factor Xa evokes a proinflammatory response in endothelial cells, which requires both its catalytic and γ-carboxyglutamic acid-containing domain. The receptor system involved in these responses induced by factor Xa remains to be established. ***The Journal of Immunology*****, 1998, 161: 4318–4324.**

The serine protease factor Xa plays a central role in the coagulation cascade, linking the extrinsic and intrinsic pathways by catalyzing the conversion of prothrombin to thrombin on vascular cell surfaces (1). Once generated, factor Xa forms the Ca^{2+}- and phospholipid-dependent prothrombinase complex with cofactor V/Va on activated platelets, monocytes, or endothelial cells (2, 3). In addition, factor Xa has been shown to mediate a variety of other biologic effects such as stimulation of lymphocyte proliferation in the presence of accessory mediators (4) and induction of mitogenesis of endothelial and smooth muscle cells (5–7). In a recent study, factor Xa has also been found to trigger acute inflammatory responses in vivo (8). Many of these cellular responses are reported to be mediated through the binding of factor Xa with the specific membrane receptor effector cell protease receptor-1 (EPR-1)[3] (5, 9, 10).

It has been well established that the inflammatory and coagulation pathways are invariably linked. Various serine proteases trigger a diversity of proinflammatory responses, in addition to their critical role in coagulation and fibrinolytic processes. For instance, thrombin and urokinase interact with specific receptors and elicit typical inflammatory responses, characterized by leukocyte migration and chemotaxis as well as adhesion to endothelium (11–15). Since factor Xa may also function as a mediator of inflammation, we aimed to search for potential proinflammatory effects of factor Xa on endothelial cells. To this end, we determined the capability of factor Xa to induce the production of the cytokines IL-6, IL-8, and monocyte chemotactic protein-1 (MCP-1), as well as the surface expression of the cell adhesion molecules E-selectin, ICAM-1, and VCAM-1, by HUVEC. In addition, we examined the structural requirements of factor Xa necessary for endothelial cell activation. Since a few studies have underscored the involvement of EPR-1 in the action of factor Xa on several cell types, we investigated its participation in the factor Xa-induced proinflammatory responses on endothelial cells.

Materials and Methods

Materials

Human thrombin (3120 NIH U/mg protein), heparin, FMLP, and LPS (from *Escherichia coli* serotype 055:B5) were all obtained from Sigma (St. Louis, MO). Hirudin and human rTNF-α were provided by BASF/Knoll (Ludwigshafen, Germany). Recombinant IL-1α and the IL-1 receptor antagonist (IL-1Ra) were kind gifts, respectively, of Dr. Gillis (Immunex, Seattle, WA) and Dr. Vannice (Amgen, Boulder, CO). Factor Xa substrate (Pefachrome Xa) was obtained from Pentapharm (Basel, Switzerland), and thrombin-cleavable substrate (S2238) from KabiVitrum (Stockholm, Sweden). Factor X was purified from bovine and human plasma as described (16) and activated with Russell's viper venom according to the method of Fujikawa et al. (17). Factor Xa activity was measured by the ability to hydrolyse Pefachrome Xa. Purity was controlled by SDS-PAGE and staining with Coomassie brilliant blue. Modified factor Xa, lacking the γ-carboxyglutamic acid (gla) residues (factor Xa-gla), was a kind gift from Drs. J. Rosing and G. Tans (Department of Biochemistry, University of Maastricht) and was prepared as described by Morita and Jackson (18). The

Departments of *Surgery, †Biochemistry, and ‡Human Biology, University of Maastricht, Maastricht, The Netherlands

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[1] Address correspondence and reprint requests to Dr. N. H. M. Senden, Department of Surgery, University of Maastricht, P.O. Box 616, 6200 MD Maastricht, The Netherlands. E-mail address: n.senden@ah.unimaas.nl

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[3] Abbreviations used in this paper: EPR-1, effector cell protease receptor-1; MCP-1, monocyte chemoattractant protein-1; PMN, polymorphonuclear leukocyte; ATIII, antithrombin III; IL-1Ra, IL-1 receptor antagonist; factor Xa gla, factor Xa lacking the γ-carboxyglutamic acid residues; DEGR, dansyl-glutamylglycyl-L-arginine; PMN, polymorphonuclear leukocytes.

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concentration of factor Xa-gla was calculated from its activity toward Pefachrome Xa, assuming identical amidolytic activity as factor Xa. Active site-inhibited factor Xa was obtained by incubation of native factor Xa with dansyl-glutamylglycyl-L-arginine (DEGR) chloromethyl ketone (Calbiochem, La Jolla, CA), as described previously (19). Antithrombin III (ATIII) was obtained from bovine plasma and purified to homogeneity according to the method of Thaler et al. (20). Incubation of factor Xa with equimolar ATIII resulted in complete loss of factor Xa coagulant activity. Factor Xa and ATIII were tested for LPS activity by the *Limulus* amebocyte lysate assay according to the manufacturer's instructions (Endosafe, Charleston, SC) and were found to contain <10 pg/ml LPS.

The anti-EPR-1 mAbs 9D4 and B6, shown to bind to the EPR-1 sequence Pro^{120}-Ala^{154}, representing the factor Xa-binding site on EPR-1, the factor X peptide Leu^{83}-Phe^{84}-Thr^{85}-Arg^{86}-Lys^{87}-Leu^{88}-(Gly), representing the interconnecting epidermal growth factor sequence in factor Xa, which mediates ligand binding to EPR-1, and its control scrambled variant Lys-Phe-Thr-(Gly)-Arg-Leu-Leu were kindly provided by Dr. D. C. Altieri (Department of Pathology, Yale University School of Medicine, New Haven, CT) (21, 22). The partially overlapping peptides S^{123}PGKPGNQNSKNEPP^{137} (AG1) and P^{136} PKKRERERSSHCYP^{150} (AG2), corresponding to the sequences of EPR-1 implicated in interaction with factor Xa (21), were synthesized by Dr. J. W. Drijfhout (Department of Immunohematology, Academic Hospital, Leiden, The Netherlands). Control mAb CB1, produced by the cell line MOPC21, was obtained from Celltech (Berkshire, U.K.). Fibronectin was kindly provided by Dr. J. van Mourik (CLB, Amsterdam, The Netherlands). RPMI 1640 and human endothelial serum-free medium with L-glutamine were obtained from Life Technologies (Paisley, U.K.); endothelial cell growth supplement was purchased from Becton Dickinson (Bedford, MA); and bovine calf serum was obtained from HyClone (Logan, UT). Human serum, obtained after informed consent from healthy donors at the Red Cross Blood Bank Zuid Limburg (Maastricht, The Netherlands), was pooled and sterilized by filtration through an 0.22-μm pore size filter (Millipore, Bedford, MA). The serum was heated at 56°C for 30 min before storage at 4°C.

Cell culturing and stimulation procedures

HUVEC were isolated from fresh umbilical cords by treatment with collagenase type I (Sigma) and were seeded into fibronectin-coated tissue culture flasks (Costar, Cambridge, MA) in RPMI 1640 supplemented with 10% human serum, 10% bovine calf serum, 50 g/ml heparin, 30 g/ml endothelial cell growth supplement, 2 mM L-glutamine, and antibiotics. For stimulation experiments, endothelial cells of passages 1 through 4 were trypsinized, seeded at a density of 10^4 cells/well, and cultured for 2 to 3 days to confluence in fibronectin-coated 96-well flat-bottom tissue culture plates in air containing 5% CO_2 at 37°C. Before stimulation, the cells were washed extensively and placed in serum-free culture medium. Stimulation of the cells was initiated by adding 100 μl of fresh serum-free medium containing the test reagents. In experiments designed to test inhibition of factor Xa, Xa was preincubated with the inhibitors for 40 min before it was added to the cells. In the experiments to elucidate the possible intermediate role of IL-1α or TNF-α, IL-1Ra or TNF-neutralizing mAb 61E71 (23) were added simultaneously with factor Xa, IL-1α, or TNF-α to the cells. For studies to investigate EPR-1 involvement cells were, respectively, preincubated for 30 min and 1 h, with increasing concentrations (0.25–1 mM) of the factor X-derived peptide Leu^{83}-Leu^{88} and its scrambled control or with various dilutions (25–100 μg/ml) of the anti-EPR-1 mAbs 9D4 and B6 or the control mAb CB1, before addition of factor Xa (5–40 nM) to the cells. In addition, for peptide competition experiments with the peptides AG1 and AG2, factor Xa was preincubated with increasing concentrations (0.25–1 mM) of these peptides for 15 min before addition to HUVEC monolayers. Culture supernatants were harvested at the end of the incubation period and stored at −20°C until use for determination of cytokine levels by ELISA. The cells were fixed for determination of adhesion molecule expression.

Normal human polymorphonuclear leukocytes (PMNs) were isolated from freshly drawn heparin-anticoagulated blood from healthy donors by means of Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation, followed by lysis of the contaminating erythrocytes in an isotonic ammonium chloride solution (24). PMNs were then resuspended in RPMI 1640 supplemented with 0.1% BSA and stimulated with 1 μM FMLP before addition to HUVEC for the adhesion assay.

Cytokine assays

Cytokine levels in culture supernatants were determined using a previously described sandwich ELISA for IL-6 (25) and a newly developed ELISA for IL-8. ImmunoMaxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with murine anti-IL-6 mAb 5E1 or a newly generated IL-8-specific murine mAb (IgG1), designated HM-7. Human rIL-6, kindly provided by Dr. W. Sebald (Physiologisch-Chemisches Institut der Universität, Würzburg, Germany), and human rIL-8, kindly provided by Dr. I. Lindley (Novartis Forschungs Institute, Vienna, Austria), were used for standard titration curves. Samples were tested in quadruplicate, diluted in 0.1% (w/v) BSA in PBS, and incubated for 2 h at room temperature. Biotinylated polyclonal rabbit Ig to human IL-6 or IL-8 were incubated at room temperature for 1 h, followed by five washings in 0.1% (w/v) Tween 20 in distilled water and a 1-h incubation with peroxidase-labeled streptavidin (Zymed, San Francisco, CA). Peroxidase activity was determined by addition of the substrate tetramethylbenzidine. The reaction was stopped after 15 min with 1 M H_2SO_4, and quantitation was performed by photospectrometry at 450 nm. MCP-1 concentrations were determined using a human MCP-1 ELISA test kit, kindly provided by HBT (Uden, The Netherlands). The lower detection limit of the immunoassays was 10 pg/ml.

Adhesion molecule detection assays

Supernatants from activated HUVEC were collected, and the endothelial cells were rinsed three times with PBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS/Ca/Mg), and fixed with 0.025% (w/v) glutaraldehyde (Serva, Heidelberg, Germany) at room temperature for 15 min. The fixed cells were rinsed three times with PBS/Ca/Mg, then incubated with 1% (w/v) BSA in PBS and analyzed for cell surface expression of E-selectin, ICAM-1, and VCAM-1. These proteins were detected, respectively, by 1 h incubation with biotinylated mAb ENA-2 (26), biotinylated mAb HM-1 (27), or mAb 1G11, the latter a kind gift from Dr. D. O. Haskard (Department of Rheumatology, Hammersmith Hospital, London, U.K.) (28, 29). After three washings in 0.1% (w/v) BSA in PBS, the primary Abs were detected by peroxidase-labeled streptavidin (Zymed) or peroxidase-conjugated goat anti-mouse IgG (Jackson, West Grove, PA) by incubation for 1 h. As negative controls, the primary Abs were replaced by 0.1% BSA in PBS or by a nonrelevant antiserum. All Abs were diluted in 0.1% BSA in PBS. Peroxidase activity was determined as described above. To correct for differences in cell numbers between individual wells, we routinely performed a crystal violet staining in each well, after the ELISA test. The culture plates were rinsed in distilled water and dried overnight. Cell nuclei were stained with 5 mM crystal violet (50 μl/well; Merck (Darmstadt, Germany) for 30 min. After rinsing in distilled water, the crystal violet was solubilized from the nuclei with 96% ethanol by shaking the plates for 10 min. Absorbance was measured by photospectrometry at 590 nm.

Adhesion assay

HUVECs (5×10^4 cells/well) were plated into fibronectin-coated 24-well tissue culture plates (Costar). When monolayers were confluent, the cells were rinsed thoroughly in RPMI 1640 and stimulated with 160 nM factor Xa or 0.2 nM TNF in serum-free culture medium. After 7 h of incubation, the cells were washed once with medium and incubated for 15 min at 37°C with PMNs (4×10^5 cells/well) in a total volume of 500 μl/well. At the end of the incubation period, the nonadherent cells were removed by washing twice with 0.1% BSA in RPMI 1640. Adherent cells were lysed with 200 μl of 0.5% Triton X-100 (Sigma) by shaking for 10 min. Myeloperoxidase activity of the adherent cells was measured by adding 200 μl of 0.04% tetramethylbenzidine. The reaction was terminated after 20 min by adding 50 μl of 5 M H_2SO_4, and the absorbance was determined at 450 nm. A standard curve was prepared by dilution of a sample of known PMN content.

Statistical analysis

Data in the figures are presented as mean ± SEM of one representative experiment. Differences between the various treatments were tested by Student's *t* test and were considered statistically significant at $p < 0.05$.

Results

Factor Xa induces cytokine production

Treatment of HUVEC with bovine factor Xa resulted in a significantly increased release of the cytokines IL-6, IL-8, and MCP-1 into the culture supernatants. Cytokine production was detectable at 5 nM factor Xa and increased with the concentration of factor Xa (Fig. 1, *A–C*). Similar concentration-response curves of cytokine production were obtained with human factor Xa (data not shown). While the amount of IL-8 released under the influence of 80 nM factor Xa was almost equal to that released by 0.1 nM TNF-α, the production of IL-6 and MCP-1 was about two to three times lower in comparison with TNF-α (data not shown). In the

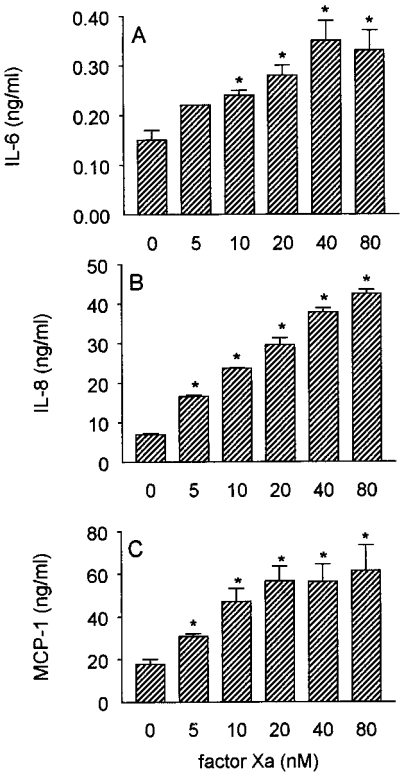

FIGURE 1. Concentration-dependent release of the proinflammatory cytokines IL-6, IL-8, and MCP-1 by factor Xa. HUVEC grown to confluence were incubated in serum-free medium with factor Xa (5–80 nM) for 20 h. Supernatants were collected and assayed for IL-6 (*A*), IL-8 (*B*), or MCP-1 (*C*) content by ELISA. Data represent mean values ± SEM of quadruplicate determinations from a representative experiment. Four sets of independent experiments were performed. Error bars were omitted if they fell within the symbol. Asterisks indicate a statistically significant difference compared with controls ($p < 0.01$).

next series of experiments, time-response curves of IL-8 production by factor Xa (10 and 80 nM) or TNF-α (0.1 nM) were determined (Fig. 2*A*). Elevated IL-8 levels became apparent as early as 4 h after stimulation with 10 or 80 nM factor Xa, and the levels continued to increase up to 24 h. The kinetics of TNF-α-induced IL-8 production follow the same pattern. After 24 h of stimulation with 80 nM factor Xa or 0.1 nM TNF-α, the levels of IL-8 in the culture media were 75 and 94 ng/ml, respectively. In addition, no disparity was observed between the kinetics of factor Xa- and TNF-α-induced production of IL-6 and MCP-1 (data not shown).

Since under physiologic conditions factor Xa becomes inhibited by protease inhibitors, we considered it important to investigate cytokine release also after short term exposures. Knowing that thrombin leads to induction of IL-8 and MCP-1 transcripts within 2 to 3 h (30, 31), we incubated HUVEC with factor Xa (40 nM) or thrombin (10 nM) for 30 or 120 min. Subsequently, the cells were maintained in factor Xa- or thrombin-free culture medium up to 20 h, after which time IL-8 and IL- 6 levels in the culture supernatants were assayed (Fig. 2*B*). IL-8 release was already increased by 30% after 30 min and by 100% after 120 min of stimulation (Fig. 2*B*). Enhanced IL-6 levels (twofold increase) became apparent after 120 min of stimulation with factor Xa (data not shown). Thrombin (10 nM) stimulation during 30 min evoked a threefold increase in IL-8 release (Fig. 2*B*). Measurements of the amount of active factor Xa showed that, after 120 min of incubation, 60% of its initial concentration could be recovered from the culture supernatant.

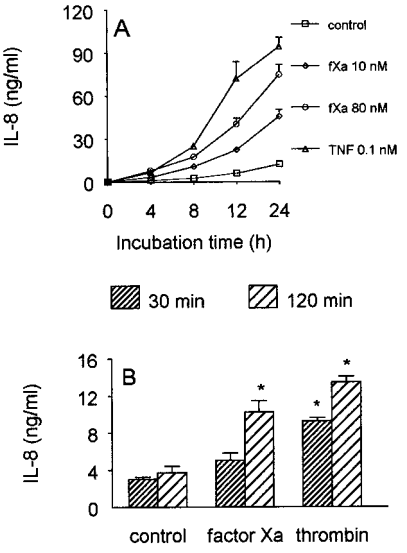

FIGURE 2. Time-dependent induction of IL-8 production by factor Xa and TNF-α. *A*, Confluent monolayers of HUVEC were stimulated with factor Xa (10 and 80 nM) or TNF-α (0.1 nM), and supernatants taken at 4, 8, 12, or 24 h were assayed for IL-8 content by ELISA. Data are expressed as mean values ± SEM of quadruplicate determinations from one experiment; at least four experiments were performed. Error bars were omitted if they fell within the symbol. *B*, HUVEC were exposed to medium alone (control), factor Xa (40 nM), or thrombin (10 nM) during a short term period of 30 or 120 min. Supernatants were collected, and the cells were washed and cultured in serum-free medium without proteases for up to 20 h. Values are mean ± SEM of quadruplicate determinations. Asterisks indicate a statistically significant difference with control cultures ($p < 0.01$).

Factor Xa induces expression of adhesion molecules

Effects on expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1 were studied in long term incubations with 5 to 160 nM factor Xa. Surface expression of E-selectin, which is not constitutively present on resting HUVEC, was measured after 5 h, whereas ICAM-1 and VCAM-1 expression were evaluated after 20 h incubation. Expression of E-selectin became apparent after stimulation with 5 to 40 nM factor Xa (Fig. 3*A*). It increased upon more prolonged exposure (8–12 h) to factor Xa, but then decreased again after 24 h of stimulation (Fig. 4). Incubation with factor Xa (40–160 nM) during 20 h resulted in a threefold increased expression of both ICAM-1 and VCAM-1 (Fig. 3, *B* and *C*).

The property of the adhesion molecules to mediate leukocyte adhesion to endothelial cells was evaluated by an adhesion assay using PMNs. Binding of PMNs to factor Xa treated endothelial cells was significantly up-regulated as compared with binding to untreated cells (Fig. 5), indicating the significance and bioactivity of the detected adhesion molecule expression.

Factor Xa exerts direct proinflammatory effects on endothelial cells

We used several approaches to identify factor Xa as the actually responsible inducer of cytokine production and adhesion molecule expression. First, control experiments using the *Limulus* amebocyte lysate assay showed that <10 pg/ml LPS was present in the factor Xa preparations, in accordance with the observation that after heating (100°C, 5 min), factor Xa failed to induce IL-8 production (Table I). Moreover, an LPS-induced response could be excluded, since addition of polymyxin B (10 μg/ml) to the culture medium did not affect the factor Xa-induced E-selectin expression, while LPS-mediated E-selectin expression was inhibited (Table II). To exclude a role of contaminating thrombin, the previous

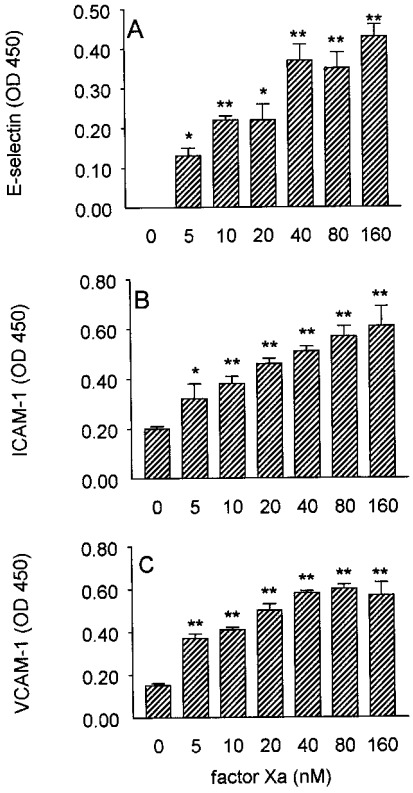

FIGURE 3. Factor Xa-induced expression of adhesion molecules. Effects are shown of different concentrations factor Xa (5–160 nM) on expression of E-selectin (*A*), ICAM-1 (*B*), or VCAM-1 (*C*). After 5 h (E-selectin) or 20 h (ICAM-1 and VCAM-1) of stimulation, supernatants were collected and the cells were fixed with glutaraldehyde. Adhesion molecule expression was examined as indicated in *Materials and Methods*. Data are the mean ± SEM of quadruplicate assays and are the results of a representative experiment. Four experiments were performed with different cell preparations. Asterisks indicate a statistically significant difference compared with controls (*, $p < 0.05$; **, $p < 0.01$).

experiments had been conducted in serum-free medium to prevent the possibility of thrombin formation from prothrombin. That thrombin was completely absent was confirmed by the inability of the culture supernatants to cleave the chromogenic thrombin substrate S2238 (data not shown). Furthermore, addition of the thrombin-specific inhibitor hirudin had no effect on the factor Xa-induced production of IL-6, IL-8, and MCP-1 nor on the expression of E-selectin, ICAM-1, and VCAM-1, regardless of the factor Xa

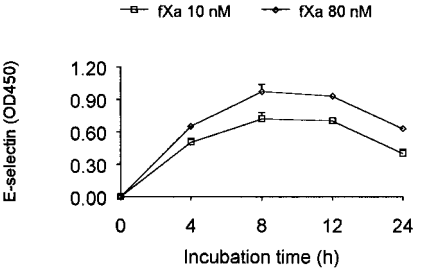

FIGURE 4. Kinetics of E-selectin expression induced by factor Xa. HUVEC were incubated for the time intervals indicated with different concentrations of factor Xa (10 and 80 nM). Expression of E-selectin by unstimulated cells remained below the detection limit. Data are expressed as mean ± SEM of quadruplicate determinations. Four experiments were performed with different cell preparations.

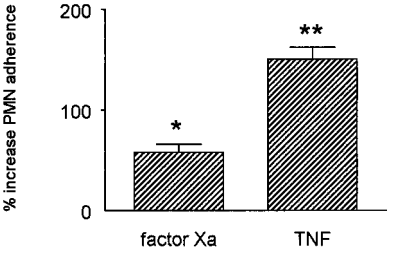

FIGURE 5. Adherence of activated PMNs to HUVEC. HUVEC were incubated for 7 h with 160 nM factor Xa or 0.1 nM TNF-α and incubated after washing with FMLP-activated PMNs. Adherent cells were detected using myeloperoxidase activity as indicated in *Materials and Methods*. The results are expressed as the percentage increase of PMN adherence compared with untreated HUVEC (mean ± SEM of five determinations). Asterisks indicate a significant increase compared with untreated HUVEC (*, $p < 0.01$; **, $p < 0.001$).

concentration used, while thrombin-induced effects were impaired (Tables I and II). ATIII, a physiologic inhibitor of both thrombin and factor Xa, completely abolished the factor Xa-induced cytokine production or adhesion molecule expression (Tables I and II). Neither ATIII nor hirudin inhibited TNF-α-stimulated IL-8 production or E-selectin expression (Tables I and II).

Moreover, the direct proinflammatory effects of factor Xa on HUVEC were confirmed using the IL-1Ra and a TNF-neutralizing Ab, 61E71, to rule out an intermediate role of IL-1α or TNF-α. Neither IL-1Ra nor the anti-TNF mAb affected the expression of E-selectin (Fig. 6) or the production of IL-8 (data not shown) induced by factor Xa. IL-1α- and TNF-α-mediated effects on E-selectin expression and IL-8 production were completely inhibited by IL-1Ra and the anti-TNF mAb, respectively.

Table I. *Specificity of factor Xa-mediated IL-8 production*[a]

Activation Conditions	IL-8 (ng/ml)
Expt. 1	
Control	6.91 ± 0.32
fXa, 5 nM	16.57 ± 0.29
fXa, 80 nM	42.50 ± 1.06
fXa, 5 nM + ATIII	7.59 ± 0.37
fXa, 80 nM + ATIII	8.17 ± 0.47
ATIII	6.61 ± 0.21
Expt. 2	
Control	10.81 ± 2.37
fXa, 5 nM	29.99 ± 2.83
fXa, 80 nM	40.67 ± 1.21
fXa, 5 nM + hirudin	26.64 ± 2.30
fXa, 80 nM + hirudin	41.29 ± 1.47
Thrombin	43.50 ± 1.66
Thrombin + ATIII	13.29 ± 2.16
Thrombin + hirudin	12.91 ± 0.11
TNF-α	27.26 ± 0.64
TNF-α + ATIII	29.55 ± 0.93
TNF-α + hirudin	24.38 ± 2.24
Expt. 3	
Control	1.10 ± 0.06
fXa, 80 nM	9.60 ± 0.52
fXa, 80 nM heated	1.56 ± 0.1

[a] HUVEC were stimulated during various time periods with factor Xa (fXa; 5 or 80 nM), thrombin (10 nM), or TNFα (0.01 nM). Incubations contained ATIII (160 nM) or hirudin (5 U/ml), as indicated. Where indicated, factor Xa was heated at 100°C for 5 min. Culture supernatants were collected after 20 h (Expts. 1 and 2) or after 5 h (Expt. 3) and assayed for IL-8 content. Data represent mean values ± SEM of quadruplicate determinations and are representative of at least three independent experiments. In the same experiments, in addition to IL-8 the levels of MCP-1 and IL-6 in the supernatants were also determined; results similar to those shown here were obtained for IL-8 production.

Table II. *Specificity of factor Xa-mediated E-selectin expression*[a]

Activation Conditions	E-selectin (OD 450)
Expt. 1	
Control	0.12 ± 0.02
fXa, 10 nM	0.33 ± 0.01
fXa, 80 nM	0.52 ± 0.02
fXa, 10 nM + ATIII	0.12 ± 0.02
fXa, 80 nM + ATIII	0.12 ± 0.01
ATIII	0.11 ± 0.01
Thrombin	0.75 ± 0.03
Thrombin + ATIII	0.11 ± 0.01
fXa, 10 nM + hirudin	0.36 ± 0.04
fXa, 80 nM + hirudin	0.49 ± 0.01
Hirudin	0.13 ± 0.01
Thrombin + hirudin	0.12 ± 0.00
TNF-α	1.03 ± 0.02
TNF-α + ATIII	0.97 ± 0.02
TNF-α + hirudin	0.94 ± 0.02
Expt. 2	
Control	0.13 ± 0.01
Control + polymyxin B	0.13 ± 0.01
fXa, 40 nM	0.72 ± 0.07
fXa, 40 nM + polymyxin B	0.71 ± 0.08
LPS	0.47 ± 0.06
LPS + polymyxin B	0.15 ± 0.03

[a] HUVEC were stimulated during various time periods with factor Xa (fXa; 5–80 nM), thrombin (10 nM), TNF-α (0.1 nM), or LPS (0.1 μg/ml). Incubations contained ATIII (160 nM), hirudin (5 U/ml), or polymyxin (10 μg/ml), as indicated. Cells were fixed after 4 h and examined for E-selectin expression. Data represent mean values ± SEM of quadruplicate determinaitons and are representative of at least three independent experiments. In similar experiments, expression levels of ICAM-1 and VACM-1 were determined after 20-h incubations. These measurements gave similar results as given here for E-selectin expression.

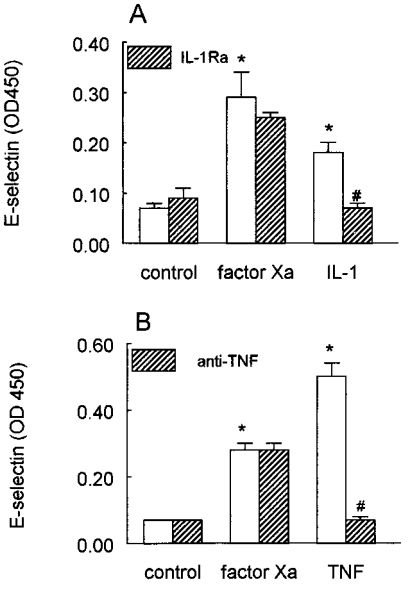

FIGURE 6. Lack of involvement of IL-1 or TNF-α on factor Xa-induced E-selectin expression. HUVEC were incubated in the absence of presence of IL-1Ra (10 μg/ml; *A*) or 61E71 (10 μg/ml; *B*) with factor Xa (80 nM), IL-1α (1 U/ml), or TNF-α (0.1 nM). After a 7-h incubation period, cells were fixed with glutaraldehyde and assayed for E-selectin expression. Data are represented as the mean ± SEM of quadruplicate determinations and are the results of one representative experiment. Three experiments were performed with different cell preparations. *, Statistically significant difference compared with controls ($p < 0.01$); #, statistically significant difference compared with cultures without IL-1Ra or 61E71 ($p < 0.01$).

Importance of the catalytic and gla domains of factor Xa for stimulation of cytokine production

To obtain information about which characteristic features of factor Xa, such as its enzymatic activity and its Ca^{2+}-membrane binding properties, are neccesary for the observed proinflammatory responses, we used two modified forms of factor Xa. First, irreversibly inactivated factor Xa was prepared by treatment with the active site inhibitor DEGR-chloromethyl ketone. The loss of enzymatic activity was verified by measuring the amidolytic activity of Pefachrome Xa, as described (19). Second, factor Xa lacking the gla residues responsible for Ca^{2+}-dependent binding (2) was tested in our HUVEC system. Both of the modified forms of factor Xa failed to induce IL-8 production (Fig. 7). Accordingly, both the active site and the gla domain seem to be required for this cytokine production.

Studies on involvement of EPR-1 in factor Xa-induced cytokine release

To address the question whether EPR-1 is involved in the factor Xa-induced cytokine production by HUVEC, we used several approaches. We applied the anti-EPR-1 mAbs B6 (data not shown) and 9D4 (5, 10), described to inhibit the binding of ^{125}I-labeled factor Xa to HUVEC and EPR-1 CHO-transfected cells in our assays. No inhibitory effect of 9D4, in a concentration range from 25 to 100 g/ml, was found on the IL-8 production induced by human or bovine factor Xa (5–40 nM; Fig. 8*A*). In addition, in peptide competition experiments, neither the factor X peptide Leu^{83}-Leu^{88} (0.25 -1 mM) (Fig. 8*B*) nor the EPR-1 derived peptide AG1 (data not shown) were effective in inhibiting factor Xa-dependent IL-8 production. Neither the EPR-1 mAbs nor the different peptides by themselves caused stimulation of IL-8 production.

Discussion

It is generally recognized that activation of coagulation is closely linked to immune and inflammatory responses in vivo. Although inflammation constitutes a major trigger by which the hemostatic anticoagulant properties of endothelium can be modified into a procoagulant state resulting in pathologic conditions such as disseminated intravascular coagulation (32, 33), it is appreciated that the coagulation process itself is also able to induce inflammatory responses (34, 35). Several studies have shown that anticoagulant treatment not only diminishes activation of coagulation but also inhibits inflammation, indicating the extensive interplay between these processes. For instance, Carr et al. showed that tissue factor

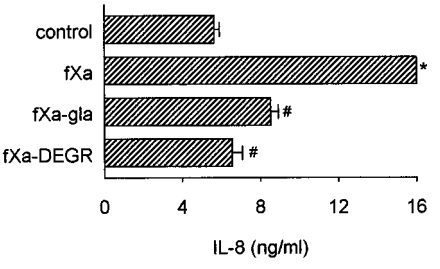

FIGURE 7. Structural requirements of factor Xa for cytokine release. HUVEC were incubated with either 40 nM factor Xa, factor Xa lacking the amino-terminal gla residues (factor Xa-gla), or factor Xa catalytically inactivated by DEGR-chloromethyl ketone treatment (factor Xa-DEGR). Culture supernatants were collected after 20 h and assayed for IL-8 content. Values are the mean ± SEM of quadruplicate determinations. Error bars were omitted if they fell within the symbol. *, Statistically significant difference compared with controls ($p < 0.001$); #, statistically significant difference compared with factor Xa treatment ($p < 0.001$).

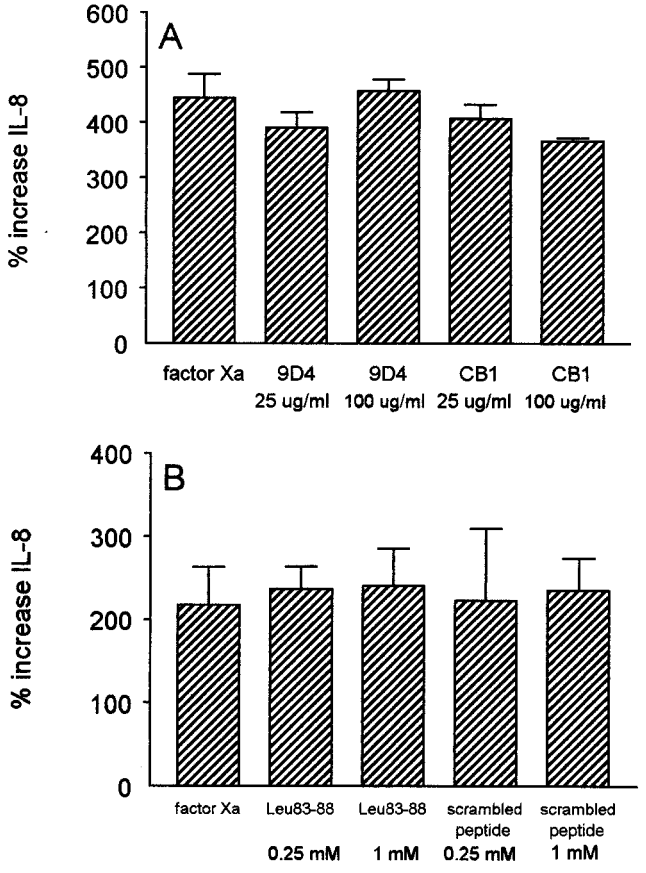

FIGURE 8. Involvement of EPR-1 in factor Xa-induced cytokine release. HUVEC were preincubated for 1 h and for 30 min, respectively, with *A*, anti-EPR-1 mAb 9D4 (25 and 100 g/ml) or the control mAb CB1 or with *B*, the factor X-peptide Leu^{83}-Leu^{88} (0.25–1 mM) or its scrambled control peptide. After preincubation, human factor Xa (40 nM) was added to the cells for 24 h in *A* and for 12 h in *B*. Supernatants were collected and assayed for IL-8 content. Values are the mean ± SEM of quadruplicate determinations and are representative of at least five experiments in each situation with either bovine or human factor Xa.

pathway inhibitor reduced the inflammatory response, as measured by plasma IL-6 levels, in a baboon model of septic shock (36). Furthermore, Hisama et al. described that ischemia-reperfusion-induced inflammatory responses, characterized by cytokine production and tissue accumulation of neutrophils, in a rat liver ischemia-reperfusion model, were significantly reduced by anticoagulant treatment, suggesting a role of microthrombus formation in the generation of inflammation after ischemia-reperfusion (37).

Recently, it has become evident that, in addition to their critical role in coagulation and fibrinolytic processes, plasma serine proteases trigger a diversity of cellular responses, including inflammatory reactions. For instance, it is well known that thrombin acts as a potent proinflammatory agonist of endothelial cells. Thrombin not only increases vascular permeability, but also stimulates attraction and binding of neutrophils and monocytes to the endothelium by induction of cytokine release and expression of adhesion molecules, such as P- and E-selectin (30, 31, 38–41). Also, factor Xa has been shown to mediate a variety of biologic effects in addition to its function as a pivotal enzyme in coagulation. Several authors have described a role for factor Xa in mitogenesis and in lymphocyte activation (4–7). There is also evidence that factor Xa can trigger inflammatory processes (8). Injection of factor Xa in the rat paw resulted in inflammation, as estimated by edema formation, through binding and activation of mast cells. Many of these biologic effects may be mediated through the interaction of factor Xa with a specific membrane receptor, EPR-1. The present study is the first to demonstrate that factor Xa is an effective inducer of a proinflammatory response in endothelial cells. Our data show that, in HUVEC, factor Xa ranging from 5 nM and higher induce time- and concentration-dependent production of the cytokine IL-6 and the chemokines IL-8 and MCP-1 and stimulate expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1. Moreover, the factor Xa-induced adhesion molecule expression accommodated neutrophil adherence to the cells. The physiologic relevance of the proinflammatory actions of factor Xa on endothelial cells remains to be established. However, short term exposure (120 min) of HUVEC to factor Xa already stimulates considerable IL-8 and IL-6 production, and prolonged exposure leads to higher levels. In vivo, factor Xa activity is down-regulated by protease inhibitors; despite this fact, concentrations of 10 nM and higher can be reached in plasma (42). Even higher concentrations of factor Xa may be present locally, since factor Xa is generated on cells by tissue factor/factor VIIa and factor VIIIa/IXa and are stabilized on their phospholipid surface by factor Va, which may lead to protracted and intense factor Xa activity on these cells. Also, factor Xa may act synergistically with other activators, a concept that is the focus of current investigations.

The effects of factor Xa closely resemble the well-known inflammatory actions of thrombin. The effects were not mediated by contamination with or formation of thrombin, but instead were direct consequences of factor Xa interaction with endothelial cells, because: 1) thrombin amidolytic activity was not detected after exposure of the cells to factor Xa; 2) the specific thrombin inhibitor hirudin was unable to block factor Xa-induced cytokine production and adhesion molecule expression; whereas 3) the combined factor Xa and thrombin inhibitor antithrombin III blocked all of these responses.

Furthermore, we could exclude the possibility that other activators, such as IL-1α and TNF-α, were involved in the observed actions of factor Xa, since neither an IL-1Ra nor a TNF-neutralizing Ab affected factor Xa-induced responses.

Studies to assess the structural domains of factor Xa involved reveal that its active site is required because treatment of factor Xa with the chloromethyl ketone inhibitor DEGR, which alkylates the histidine residue of the catalytic site of factor Xa, inhibits cytokine production. This is probably not due to defective binding of factor Xa to the cells, since Ambrosini and Altieri have shown that this type of active site-inhibited factor Xa had normal characteristics of binding to HUVEC (21). Our data thus support those from other authors showing that the active site of factor Xa is required for mitogen release by endothelial cells and for EPR-1-mediated proliferation of smooth muscle cells (5, 43). Furthermore, it was shown that the amino-terminal gla residues of factor Xa are required for induction of the proinflammatory responses in HUVEC. This finding is in accord with the data from Gajdusek et al. who have reported that factor Xa lacking the gla domain failed to induce mitogen release by endothelial cells (43). Occupancy of a limited number of gla residues by Ca^{2+} leads to stabilization of a specific conformational state of the protein and thus supports its binding to membrane lipids and membrane-bound factor Va (44). Although not much is yet know about the ligand requirements to activate other factor Xa receptors, our data indicate that the gla domain has a structural function that would support these interactions.

Since EPR-1 is reportedly involved in various factor Xa-mediated biologic events such as the increase of cytosolic-free calcium in single adherent T cells (4), the augmentation of CD3/T cell receptor-dependent lymphocyte proliferation (4), the costimulated

proliferation of mononuclear cells (4), and mitogenic signaling of endothelial and smooth muscle cells (5–7), we have investigated whether the proinflammatory responses evoked in HUVEC by factor Xa are also mediated by EPR-1. An inflammatory response, as measured by the formation of edema (8) is mediated by the factor X-derived peptide Leu^{83}-Leu^{88}, which mediates ligand binding to EPR-1. This peptide lacks enzymatic activity, which indicates that the active site of factor Xa was not involved in that particular inflammatory response. In contrast, our studies clearly show that the enzymatic activity of factor Xa is needed for the induction of cytokine production and adhesion molecule expression by HUVEC. Since the results of our studies indicate that EPR-1 is not involved in factor Xa-stimulated cytokine production, the exact biologic mechanism of factor Xa-induced proinflammatory responses in HUVEC needs to be determined.

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